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A short sequence immediately upstream of the internal repeat elements is critical for KSHV LANA mediated DNA replication and impacts episome persistence

Erika De León Vázquez, Franceline Juillard, Bernard Rosner, Kenneth M. Kaye *

Departments of Medicine, Brigham and Women's Hospital and Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115, United States

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus LANA (1162 residues) mediates episomal persistence of viral genomes during latency. LANA mediates viral DNA replication and segregates episomes to daughter nuclei. A 59 residue deletion immediately upstream of the internal repeat elements rendered LANA highly deficient for DNA replication and modestly deficient for the ability to segregate episomes, while smaller deletions did not. The 59 amino acid deletion reduced LANA episome persistence by ~14-fold, while sequentially smaller deletions resulted in ~3-fold, or no deficiency. Three distinct LANA regions reorganized heterochromatin, one of which contains the deleted sequence, but the deletion did not abolish LANA's ability to alter chromatin. Therefore, this work identifies a short internal LANA sequence that is critical for DNA replication, has modest effects on episome segregation, and substantially impacts episome persistence; this region may exert its effects through an interacting host cell protein(s).

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus 8 (HHV-8)) is the only gamma-2 herpesvirus that infects humans. KSHV has a causative role in Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease (Cesarman et al., 1995; Chang et al., 1994; Moore and Chang, 1995; Soulier et al., 1995). KSHV latently infects tumor cells and during latent infection only expresses a small subset of genes. Cells latently infected with KSHV maintain multiple copies of the viral genome as circular, covalently closed, extrachromosomal forms (episomes) (Cesarman et al., 1995; Decker et al., 1996). Latency-associated nuclear antigen (LANA), encoded by open reading frame 73 (ORF73) (Kedes et al., 1997; Kellam et al., 1997; Rainbow et al., 1997), is necessary and sufficient for episome persistence (Ballestas et al., 1999; Ballestas and Kaye, 2001).

There are two key components to episome persistence, DNA replication and segregation of episomes to daughter nuclei, and LANA fulfills both these functions. Both N-and C-terminal LANA are essential for episome maintenance. LANA associates with mitotic chromosomes and has two independent chromosome binding regions located in N- and C-terminal LANA (Fig. 1) (Ballestas et al., 1999; Barbera et al., 2004; Kelley-Clarke et al., 2007a, 2007b; Krithivas et al., 2002; Lim et al., 2004; Piolot et al., 2001; Szekely et al., 1999; Wong et al., 2004). N-terminal LANA is the dominant chromosome attachment region and binds mitotic chromosomes by directly interacting with histones H2A/H2B on the nucleosome surface. This interaction is essential for episome maintenance and efficient DNA replication (Barbera et al., 2004; Barbera et al., 2006; Hu et al., 2002). C-terminal LANA self-associates to bind two adjacent sites in each KSHV terminal repeat (TR) element to mediate DNA replication (Ballestas and Kaye, 2001; Cotter et al., 2001; Fejer et al., 2003; Garber et al., 2002; Garber et al., 2001; Grundhoff and Ganem, 2003; Hu et al., 2002; Komatsu et al., 2004; Lagunoff and Ganem, 1997; Lim et al., 2002). LANA mediates segregation of DNA to progeny nuclei by simultaneously binding TR DNA and mitotic chromosomes.

Expression of LANA in uninfected cells causes nuclear reorganization with release of DNA from heterochromatic areas, which can be observed in human and mouse cells (Mattsson et al., 2002; Stuber et al., 2007). These changes are more easily detected in murine cells due to the presence of pericentromeric alpha-satellite repeats that are organized into well defined heterochromatic chromocenters (Stuber et al., 2007). The LANA region exerting this effect was mapped to residues 275-331(Stuber et al., 2007).

We previously showed that, in addition to N- and C-terminal LANA, internal LANA sequence is also critical for episome persistence since fusion of N- and C-terminal LANA resulted in highly deficient episome maintenance (De Leon and Kaye, 2011a). Further, a panel of large internal deletion mutants suggested that a small region, located immediately upstream of the internal

* Correspondence to: Division of Infectious Diseases, Brigham and Women's Hospital, 181 Longwood Ave., Boston, MA 02115, United States.
Tel.: +1 617 525 4256; fax: +1 617 525 4251.
E-mail address: kkaye@rics.bwh.harvard.edu (K.M. Kaye).

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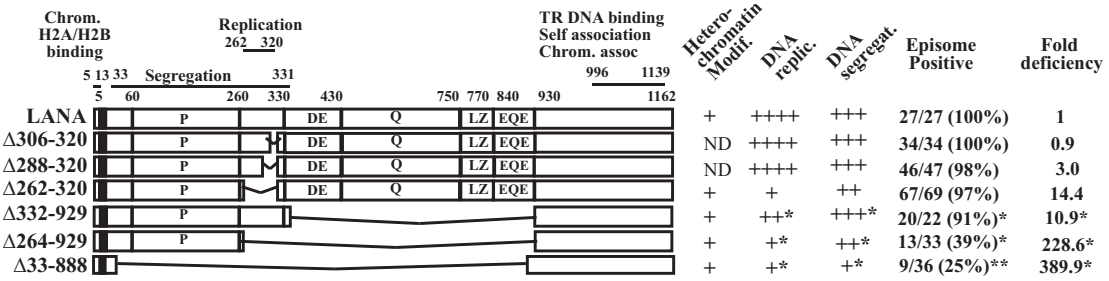


Fig. 1. Schematic diagram of KSHV LANA and deletion mutants. The proline rich region (P), aspartate and glutamate region (DE), glutamine and glutamate region (Q), glutamine and glutamate region (EQE), and putative leucine zipper (LZ) are indicated. The DE, Q, EQE and LZ regions are all comprised of repeat elements. Shaded region indicates the N-terminal nuclear localization (NLS) signal. C-terminal LANA can also localize to nuclei but an NLS has not been precisely mapped. Amino acids 5–13 mediate chromosome association through binding histones H2A/H2B. Amino acids 996–1139 have DNA binding, self-association and chromosome association functions. Capabilities for heterochromatin modification, DNA replication, DNA segregation, episome maintenance and fold deficiency for episome maintenance are summarized at the right for LANA and each mutant. Fractions are the number of G418 resistant cell lines containing episomes over the total number of G418 resistant cell lines assayed by Gardella analysis and percentages are shown in parenthesis. ND, not done. Asterisks indicate results of previous work (De Leon Vazquez et al., 2013).

repeat elements, may have a key role in episome persistence (De Leon Vazquez et al., 2013). Notably, this region overlaps with residues 275-332 that were reported to reorganize heterochromatin. Here we directly investigate the role of this region in episome maintenance. We find that this sequence is critical for DNA replication, modestly affects segregation and substantially impacts episome persistence. Although we confirmed that LANA reorganizes heterochromatic regions, LANA deleted for this small region was still capable of reorganizing heterochromatin.

Results

LANAΔ262-320 maintains the ability to release DNA from heterochromatic chromocenters

We recently showed that internal sequence exerts critical effects on LANA's ability to mediate episome persistence. N- and C-terminal LANA are essential for episome persistence. N-terminal LANA mediates mitotic chromosome attachment and C-terminal LANA binds KSHV TR DNA and also has a role in chromosome attachment. These components are essential for LANA mediated DNA replication and tethering of KSHV episomes to mitotic chromosomes, which provides a mechanism to segregate DNA to daughter nuclei. Deletion of all internal regions revealed that this sequence is also critical for episome persistence (De Leon and Kaye, 2011a). A panel of mutants deleted for large portions of the internal sequence suggested that a small internal LANA region immediately upstream of the internal repeat elements may exert an important role. LANA deleted for amino acids 332-929 (LANAΔ332-929) was 10.9 fold reduced for episome persistence efficiency, while LANA deleted for residues 264-929 (LANAΔ264-929) was reduced 228.6 fold (Fig. 1) (De Leon Vazquez et al., 2013). Here, we perform a targeted investigation of the region immediately upstream of the LANA internal repeat elements.

Previous work (Stuber et al., 2007) demonstrated that LANA reorganizes DNA staining patterns by releasing DNA from heterochromatic regions. These effects were particularly evident in murine cells, such as fibrosarcoma L cells, which contain pericentromeric DNA organized in well defined heterochromatic chromocenters. This function was mapped to LANA residues 275-332. Since these residues overlap with the LANA 264–331 sequence that were implicated as important for episome persistence, we asked whether the effect on episome persistence might be related to LANA's ability to reorganize heterochromatin.

We generated LANAΔ262-320 (Fig. 1), which is deleted for the indicated residues, and assessed its ability to reorganize heterochromatin. GFP, LANA or LANAΔ262-320 were expressed in murine L cells. GFP (green) (Fig. 2A panels a–c) did not cause rearrangement or modification of DNA (blue, or black and white) chromocenters (bright DNA foci, arrow indicates example in Fig. 2B). Consistent with previous results (Stuber et al., 2007), cells expressing LANA released DNA from chromocenters (Fig. 2A, panels d–f), causing disappearance of DNA foci, in contrast to adjacent, untransfected cells that lacked LANA expression. LANAΔ262-320 also released DNA from chromocenters (Fig. 2A, panels g–i). Fig. 2B shows a cell expressing a moderate level of LANAΔ262-320 and an adjacent cell expressing a very low level of LANAΔ262-320. Different optical slices of the cell are shown from a stack of images, which permits better definition of the bright chromocenters. The cell expressing a moderate level of LANAΔ262-320 shows release of DNA from chromocenters while the adjacent cell expressing a very low level of LANAΔ262-320 does not. These results suggest that LANA residues 262-320 are dispensable for the release of DNA from chromocenters.

It remained possible that LANA residues 321–332 exert a role in chromatin modification, since these amino acids were also implicated in the earlier work but were not deleted in LANAΔ262-320. Therefore, we transfected L cells with LANAΔ264-929, which lacks these residues, or LANAΔ332-929 which contain all but amino acid 332. Both LANAΔ332-929 (Fig. 2C) and LANAΔ264-929 (Fig. 2D) released DNA from chromocenters, indicating that residues 264-929 are dispensable for this effect. We also expressed LANAΔ33-888, which contains only N- and C-terminal LANA, in L cells, and this mutant also released DNA from chromocenters (Fig. 2E).

To further localize the LANA region(s) responsible for heterochromatin reorganization, we assessed a panel of LANA mutants for the ability to release DNA from chromocenters (Fig. 2F). GFP, LANA with an N-terminal GFP fusion (GFP LANA), or GFP LANA mutants were expressed in L cells, and the percent of GFP or GFP LANA expressing cells with reorganized heterochromatin was determined. As expected, GFP did not result in heterochromatin reorganization, while GFP LANA altered chromatin in 87.5% of cells. GFP LANAΔ33-888, comprised of N- and C-terminal LANA, released DNA from chromocenters in 67.2% of cells, modestly less than that of LANA. To investigate the role of N-terminal LANA as well as LANA 33-331, the sequence located just downstream of N-terminal LANA, we assessed GFP LANA 1-331, GFP LANA 1-32, GFP LANA 33-331, and GFP LANA 1-331 GMR (mutated at residues $_5$GMR$_7$, resulting in loss of histone H2A/H2B binding and mitotic chromosome association (Barbera et al., 2004)), which released DNA from chromocenters in 60.4%, 10.9%, 23.0%, and 32.8% of cells, respectively. These results indicated that LANA 1-32 and LANA ;33-331 appear to contain distinct functional regions capable of reorganizing heterochromatin.

Notably, LANA 33-331 overlaps with residues 275-332 identified by Stuber et al. as having a role in chromatin reorganization (Stuber et al., 2007). In addition, GFP LANA 779-1049 and GFP LANA 933-1162 altered chromatin in 16.7% and 33.3% of cells, respectively, indicating C-terminal LANA also independently can reorganize heterochromatin. Therefore, these results suggest that at least three LANA regions, LANA 1–32, LANA33-331, and C-terminal LANA, each are capable of releasing DNA from chromocenters. LANAΔ262-320 contains two of these regions, with a 59 amino acid deletion within the third region, and remained capable of reorganizing heterochromatin (Fig. 2B).

KSHV LANAΔ262-320 and LANAΔ288-320, but not LANAΔ306-320, are reduced for episome persistence

We investigated the role of the LANA residues upstream of the internal repeat elements in episome persistence. In addition to LANAΔ262-320, we constructed LANAΔ288-320 and LANAΔ306-320, which are deleted for the indicated residues. BJAB cells or BJAB cells stably expressing LANA, LANAΔ262-320 (cell line termed LANAΔ262-320Y), LANAΔ288-320 or LANAΔ306-320 (Fig. 1) were generated. Cells were transfected with p8TR, which contains eight copies of the KSHV TR's, and 3 days after transfection, seeded at 1000, 100, 10, or 1 cell/well in 96 well plates, and grown under G418 selection. From an average of three experiments, BJAB cells, which require integration of p8TR for G418 resistance, had an average G418 resistant outgrowth in 84, 15, 2, and 0 wells after seeding at 1000, 100, 10, or 1 cell/well (Fig. 3A). In contrast, LANA expressing cells had an average G418 resistant outgrowth of 96, 96, 43, and 4 wells after seeding at 1000, 100, 10, or 1 cell/well (Fig. 3A). The higher outgrowth is due to the much higher efficiency of LANA episome maintenance compared to integration. LANAΔ306-320 had G418 resistant outgrowth that was similar to that of LANA and cells grew in an average of 96, 96, 55, and 4 wells after seeding at 1000, 100, 10, or 1 cell/well (Fig. 3A). In contrast, LANAΔ288-320 was reduced for outgrowth compared to LANA, and grew in an average of 96, 76, 7, and 0 wells after seeding at 1000, 100, 10, or 1 cell/well (Fig. 3A).

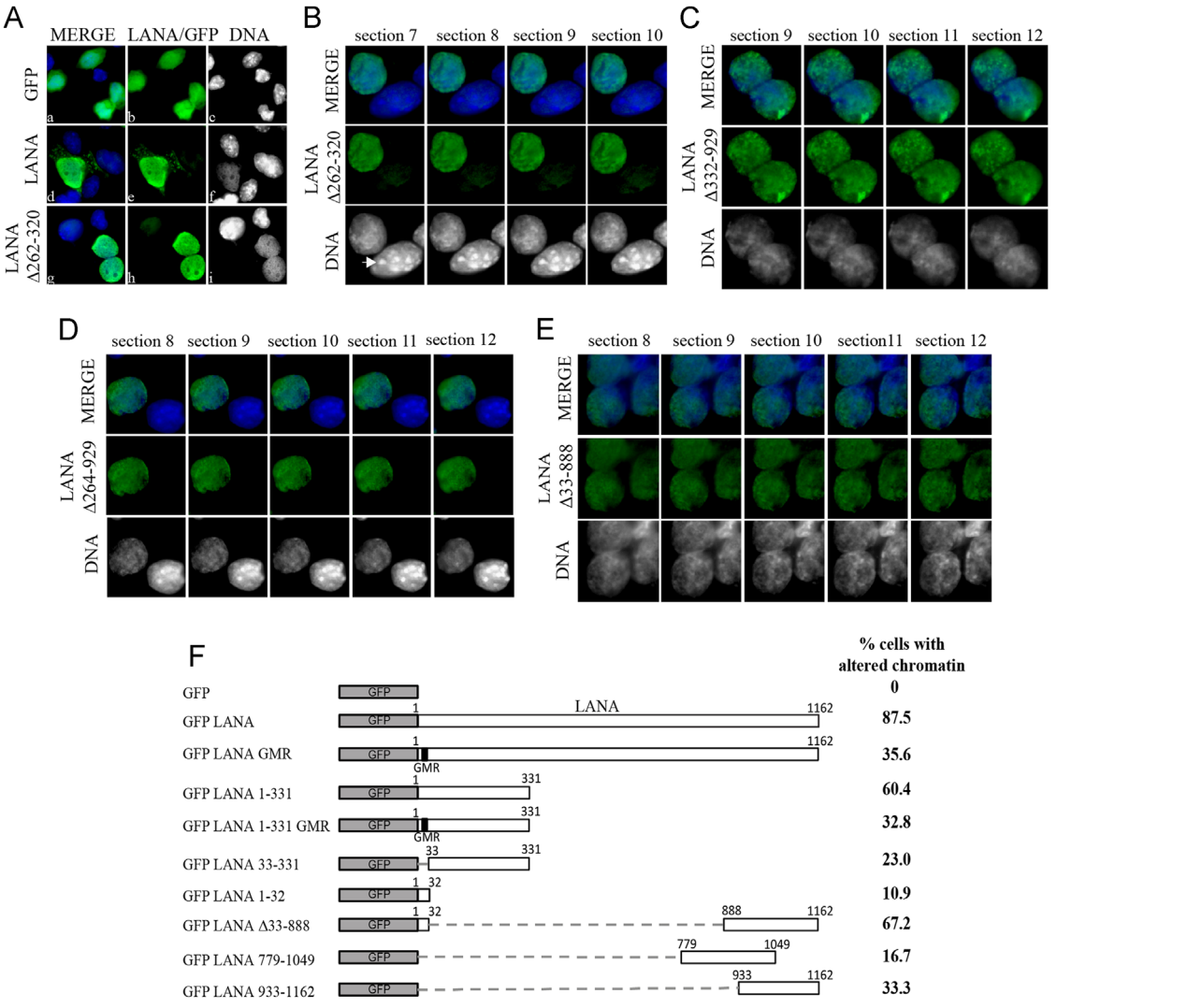


Fig. 2. LANA and LANA deletion mutants exert effects on heterochromatin organization in murine L-cells. (A) Expression of LANA (green) and LANAΔ262-320 (green), but not GFP, reorganizes DNA (blue in merge, black and white in right column) from chromocenters (bright DNA foci seen in adjacent non-transfected cells). (B) A cell expressing moderate levels of LANAΔ262-320 exhibits reorganized heterochromatin, but an adjacent cell expressing a very low level LANAΔ262-320 does not. Slices 7–10 (from a stack of 18) are shown and provide better definition of chromocenters. Arrow indicates a chromocenter. (C) LANAΔ332-929 slices 9–12 (from a total of 18) (D) LANAΔ264-929 slices 8–12 (from a total of 23) (E) LANAΔ33-888 slices (from a total of 19). LANA and LANAΔ262–320 were detected with anti-LANA antibody, which recognizes a multi-copy internal repeat element within LANA. LANAΔ332-929, LANAΔ264-929, and LANAΔ33-888, lack the internal repeat elements and were detected with anti-T7 antibody, which recognizes a copy of the T7 epitope at N-terminal LANA. Magnification, 630 × . (F) Percentage of cells with heterochromatin reorganization after expression of GFP, GFP LANA or GFP LANA mutants. At least 50 cells from random fields were assessed for each mutant from four independent transfections.

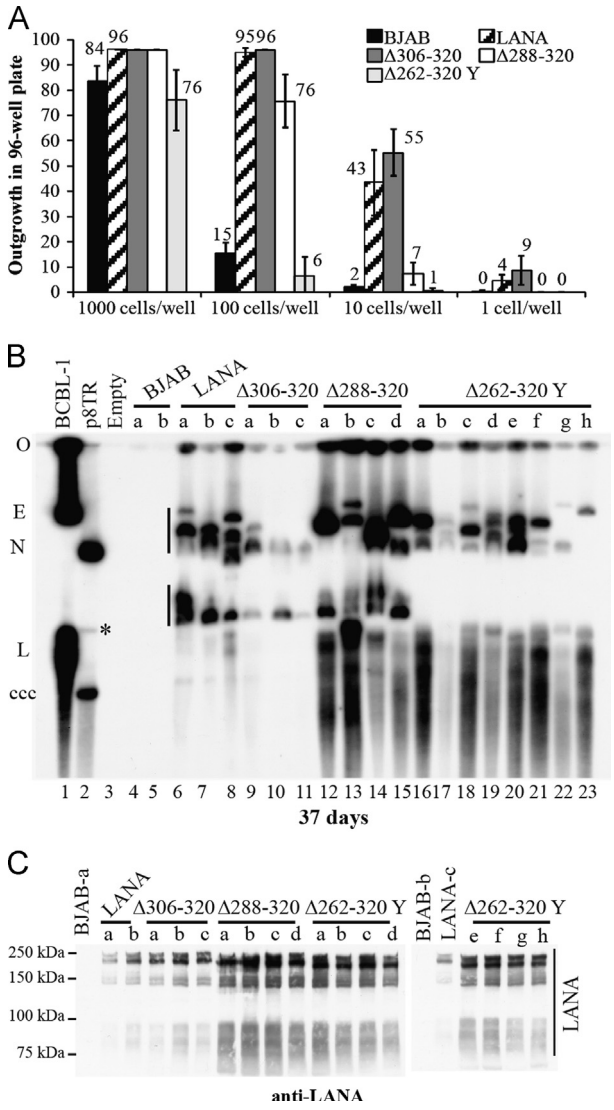


Fig. 3. LANAΔ288-320 and LANAΔ262-320Y are deficient for episome maintenance. (A) G418 resistant outgrowth of BJAB cells or BJAB cells stably expressing LANA, LANAΔ306-320, LANAΔ288-320 or LANAΔ262-320Y after p8TR transfection. Three days after transfection cells were seeded at 1000, 100, 10, or 1 cell/well and grown under G418 selection. Outgrowth was scored after 21 days of selection. Values are averages of three independent experiments, with error bars indicating the standard deviation. (B) Gardella gel analysis of G418 resistant cell lines to detect the presence of episomal DNA. BCBL-1 (a KSHV infected primary effusion lymphoma) (lane 1), naked p8TR plasmid (lane 2), G418 resistant BJAB cells (lanes 4–5), or BJAB cells stably expressing LANA (lanes 6–8), LANAΔ306-320 (lanes 9–11), LANAΔ288-320 (lanes 12–15) or LANAΔ262-320Y (lanes 16–23). G418 resistant cells run on the Gardella gel were expanded from 100 cells/well except for LANAΔ262-320Y which were from 1000 cells/well. Approximately 1.5×10^6 cells were loaded per lane. O, origin; E, BCBL-1 episome; L, linear BCBL-1 DNA resulting from lytic replication; ccc, covalently closed circular plasmid DNA (lane 2); N, nicked plasmid DNA (lane 2); asterisk indicates minor form of p8TR plasmid DNA (lane 2); vertical lines indicate faster and slower migrating forms of episomes. Results are representative of three independent experiments. (C) Western blot analysis of G418 resistant cell lines used for Gardella cell analysis. 1.5×10^6 cells were loaded per lane, and LANA detected with anti-LANA antibody. Brightness and contrast in panels B and C were uniformly adjusted using Adobe Photoshop.

LANAΔ262-320Y was further reduced for outgrowth and grew in an average of 76, 6, 1, and 0 wells after seeding at 1000, 100, 10, or 1 cell/well (Fig. 3A). Therefore, G418 resistant outgrowth was modestly less efficient for LANAΔ288-320 and highly deficient for LANAΔ262-320Y cells compared with LANA, consistent with modest and more severe episome maintenance deficiencies for LANAΔ288-320 and LANAΔ262-320Y, respectively.

We directly assessed for the presence of episomes in G418 resistant cell lines. BJAB cells or cells expressing LANA, LANAΔ306-320, LANAΔ288-320 or LANAΔ262-320Y were analyzed by Gardella analysis (Gardella et al., 1984). In a Gardella gel, live cells are loaded into gel wells and lysed *in situ*. During electrophoresis, chromosomal DNA remains at the origin while episomal DNA (as large as several hundred kilo bases) migrates into the gel. Episomal DNA is detected by Southern blot with radiolabeled probed. BCBL-1, a KSHV infected primary effusion lymphoma cell line, had a slowly migrating band representing the viral episome (Fig. 3B, lane 1; E indicates episome) and, a faster migrating band due to linear DNA that is associated with lytic replication of the virus (Fig. 3B, lane 1; L indicates linear). As expected, BJAB cells lacking LANA expression did not contain episomal DNA (Fig. 3B, lanes 4–5), while cells expressing LANA contained extrachromosomal DNA in all lanes (Fig. 3B, lanes 6–8). Cells expressing LANAΔ306-320 (Fig. 3B, lanes 9–11) also had episomal DNA in all lanes, as did LANAΔ288-320 (Fig. 3B, lanes 12–15). Unexpectedly, despite its high level deficiency in limiting dilution outgrowth (Fig. 3A), LANAΔ262-320Y had episomal DNA in all lanes (Fig. 3B, lanes 16–23).

Interestingly, the pattern of episomal DNA present in LANAΔ262-320Y lanes differed from that in the LANA, LANAΔ306-320, and LANAΔ288-320 lanes. The faster migrating episomal DNA present in LANA, LANAΔ306-320, and LANAΔ288-320 lanes (Fig. 3B, lower vertical line adjacent to lane 6) was absent in all LANAΔ262-320Y lanes. This faster migrating DNA co-migrates with a minor form of DNA seen in the naked, input p8TR plasmid DNA (Fig. 3B, lane 2, asterisk) that migrates between nicked and circular, covalently closed DNA. This episomal species has been previously observed, particularly as a predominant episomal form at very early time points of G418 selection (De Leon and E., Kaye, 2011a). Smears of even faster migrating DNA were also present, and particularly prominent in the LANAΔ288-320 and LANAΔ262-320Y lanes, and may be due to partially replicated or degraded DNA. Slower migrating episomal DNA was also present in all lanes with episomes (Fig. 3B, upper vertical line adjacent to lane 6). These bands migrate near episomal BCBL-1 DNA, which is ~200 kb. These slower migrating forms are due to recombination events including expansion of TR elements and multimers of input pTR plasmid. The proportion of slowly migrating DNA increases with increasing days of G418 selection, due to selection for these recombinants (Ballestas and Kaye, 2001; De Leon and Kaye, 2011a). Western blot analysis of cell lines (Fig. 3C) showed that all cell lines express protein at levels at least as high as LANA (Fig. 3C). Therefore, the LANA mutants maintained episomal DNA but LANAΔ262-320Y cell lines exhibited more rapid selection for slower migrating, recombinant episomes.

Cell line LANAΔ262-320Y is mildly reduced for cell growth while cell lines LANAΔ262-320A, LANAΔ262-320B, and LANAΔ262-320C grow normally

We noticed that the BJAB cell line expressing LANAΔ262-320Y, used in the above assays (Fig. 3), grew at a modestly slower rate compared to all other BJAB and BJAB cell lines expressing LANA. Therefore, we generated three additional BJAB cell lines stably expressing LANAΔ262-320, denoted LANAΔ262-320A, LANAΔ262-320B, and LANAΔ262-320C. All cell lines expressed LANAΔ262-320 at levels at least as high as LANA (Fig. 4A). We compared the growth of BJAB, LANA, LANAΔ306-320, LANAΔ288-320 and four independently derived LANAΔ262-320 cell lines in the absence of episomal DNA. Cells were seeded in 96-well plates at 1000, 100, 10, or 1 cell/well and grown in Hygromycin B containing medium, for which the cells are resistant (Fig. 4B). All cell lines grew in all wells after seeding at 1000 or 100 cells/well. However, after seeding at 10 cells/well, LANAΔ262-320Y had outgrowth in 95 wells while all

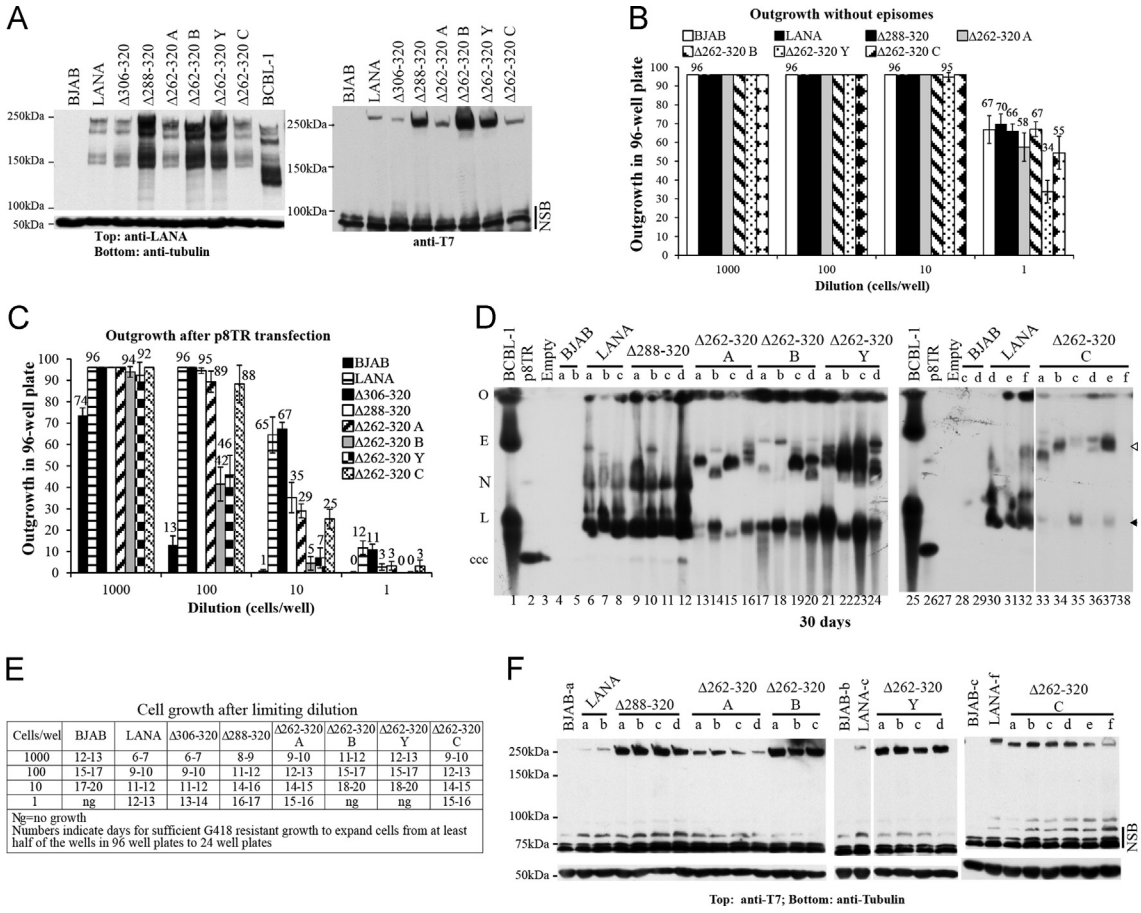


Fig. 4. LANAΔ262-320A, LANAΔ262-320B, LANAΔ262-320C, and LANAΔ262-320Y are reduced for episome persistence. (A) Western blot for LANA and LANA mutants. Lanes contain $\sim 1 \times 10^5$ cells for the anti-LANA blot or $\sim 3.5 \times 10^5$ cells for the anti-T7 blot. Anti-tubulin blot is shown at bottom left. NSB, non-specific band. (B) Limiting dilution assay of BJAB or BJAB cell lines stably expressing LANA or LANA mutants in the absence of episomal DNA. The average of three experiments with standard deviation is shown. (C) BJAB or BJAB cells stably expressing LANA, LANAΔ306-320, LANAΔ288-320, LANAΔ262-320A, LANAΔ262-320B, LANAΔ262-320C or LANAΔ262-320Y were transfected with p8TR and 3 days post-transfection seeded in 96-well plates at different concentrations and placed under G418 selection. Two independently derived LANAΔ306-320 or LANAΔ288-320 cell lines were used, and since the cell lines for each mutant behaved similarly, results were averaged together. The average of three experiments is shown. (D) Gardella gel analysis of G418 resistant cell lines. BCBL-1 (a KSHV infected primary effusion lymphoma cell line) (lanes 1, lane 25), naked p8TR plasmid (lanes 2, 26), G418 resistant BJAB cells (lanes 4–5, 28–29), BJAB cells stably expressing LANA (lanes 6–8, 30–32), LANAΔ288-320 (lanes 9–12), LANAΔ262-320A (lanes 13–16), LANAΔ262-320B (lanes 17–20), LANAΔ262-320Y (lanes 21–24), and LANAΔ262-320C (lanes 33–38). G418 resistant cells run on Gardella were expanded from 100 cells/well. Approximately 1.5×10^6 cells were loaded per lane. O, origin; E, BCBL-1 episome; L, linear BCBL-1 DNA resulting from lytic replication; ccc, covalently closed circular plasmid DNA (lanes 2, 26); N, nicked plasmid DNA (lanes 2, 26); solid and empty arrowheads indicate faster and slower migrating episomal DNA, respectively. (E) BJAB cells or BJAB cells expressing LANA or LANA mutants were assessed for rate of G418 resistant outgrowth after seeding at different cell concentrations. (F) Western blot analysis from G418 resistant cell lines used for Gardella cell analysis. Each lane contains $\sim 3 \times 10^5$ cells. LANA was detected with anti-T7 epitope HRP conjugated antibody. Bottom panel shows anti-alpha tubulin blot. Brightness and contrast in individual panels were uniformly adjusted using Adobe Photoshop. NSB, non-specific band.

other lines grew in all wells. In addition, after seeding at 1 cell/well LANAΔ262-320Y was further reduced and grew in an average of only 34 wells, while BJAB, LANA, LANAΔ288-320, LANAΔ262-320A, LANAΔ262-320B, and LANAΔ262-320C all had outgrowth in 55–70 wells. In addition to the reduced outgrowth in the number of wells, LANAΔ262-320Y consistently took ~2 days longer to grow out in the microtiter plates compared to all other cell lines. Therefore, LANAΔ262-320Y exhibited mildly reduced growth, possibly due to integration of transfected DNA at a site important for cell growth, while LANAΔ262-320A, LANAΔ262-320B, and LANAΔ262-320C grew similar to BJAB cells.

LANAΔ262-320A, LANAΔ262-320B, LANAΔ262-320C, and LANAΔ262-320Y are reduced for episome persistence

Since it was possible that the LANAΔ262-320Y's reduced growth may have affected the finding of reduced episome maintenance efficiency as assessed by limiting dilution outgrowth (Fig. 3A), we assessed LANAΔ262-320Y in parallel with the additional LANAΔ262-320 cell lines. BJAB, LANA, LANAΔ306-320, LANAΔ288-320, or LANAΔ262-320 cell lines A, B, C, or Y were transfected with p8TR DNA. Three days post-transfection cells were seeded in 96-well plates at 1000, 100, 10, or 1 cell/well, and grown under G418 selection. After seeding at 1000, 100, 10, or 1 cell/well, BJAB cells had outgrowth in 74, 13, 1, or 0 wells, respectively (Fig. 4C), while, as expected, LANA outgrowth was much more robust and occurred in 96, 96, 65, or 12 wells, respectively (Fig. 4C). LANAΔ306-320 had similar outgrowth to LANA and occurred in 96, 96, 67, and 11 wells after seeding at 1000, 100, 10, or 1 cell/well, respectively, consistent with results in Fig. 3A. Also, similar to results in Fig. 3A, LANAΔ288-320 had reduced outgrowth compared to LANA and LANAΔ306-320, and grew in 96, 95, 35, and 3 wells after seeding at 1000, 100, 10, or 1 cell/well. Outgrowth of all LANAΔ262-320 cell lines was even further reduced. LANAΔ262-320A grew in 96, 89, 29, and 3 wells, LANAΔ262-320B grew in 94, 42, 5, and 0 wells, LANAΔ262-320Y grew in 92, 46, 7, and 0 wells, and LANAΔ262-320C grew in 96, 88,

25, and 3 wells after seeding at 1000, 100, 10, or 1 cell/well, respectively (Fig. 4C). Therefore, reduction in outgrowth was greater for LANAΔ262-320B and LANAΔ262-320Y compared to LANAΔ262-320A and LANAΔ262-320C. The reduced outgrowth of LANAΔ288-320 and LANAΔ262-320 cell lines compared to that of LANA and LANAΔ306-320 was also reflected in an increased number of days for sufficient outgrowth to allow transfer from 96 to 24 well plates (Fig. 4E). Therefore, LANAΔ288-320 and LANAΔ262-320 cell lines all had reduced outgrowth, consistent with deficient episome persistence in these mutants, and LANAΔ262-320 was more deficient than LANAΔ288-320. Of the LANAΔ262-320 cell lines, LANAΔ262-320Y and LANAΔ262-320B were the most reduced. Of note, LANAΔ262-320B and LANAΔ262-320Y had higher protein expression levels than LANAΔ262-320A and LANAΔ262-320C (Fig. 4A), and it is possible that this higher expression may relate to the lower outgrowth.

G418 resistant cell lines were assessed for the presence of episomes. BJAB cells or BJAB cells expressing LANA, LANAΔ306-320, LANAΔ288-320, LANAΔ262-320 were analyzed by Gardella gels. As expected, BJAB cells lacked episomal DNA (Fig. 4D, lanes 4–5, 28–29), while LANA had episomal DNA in all lanes (Fig. 4D, lanes 6–8, 30–32). As observed earlier (Fig. 3B), all LANAΔ288-320 lanes had episomal DNA (Fig. 4D, lanes 9–12). LANAΔ262-320A (Fig. 4D, lanes 13–16), LANAΔ262-320B (Fig. 4D, lanes 17–20), and LANAΔ262-320Y (Fig. 4D, lanes 21–24) had episomes in all lanes, while LANAΔ262-320C had episomes in five of six lanes (Fig. 4D, lanes 33–38). In a total of seven experiments, LANA had episomes in 27 of 27 (100%) cell lines, in five experiments LANAΔ306-320 had episomes in 34 of 34 (100%) cell lines, in 7 experiments LANAΔ288-320 had episomes in 46 of 47 (98%) cell lines, and in seven experiments the combined LANAΔ262-320 cell lines had episomes in 67 of 69 (97%) lanes (Fig. 1). Western blot analysis of cell lines (Fig. 4F) shows all cell lines from Gardella gels express LANA at levels at least as high as wild-type LANA.

Differences in the patterns of episomal DNA migration in these Gardella gels (Fig. 4D) were again noted for LANAΔ262-320 cell lines compared with LANA. Most of the LANA episomal DNA migrated near the p8TR ccc DNA (Fig. 4D, lanes 6–8, 30–32, solid arrowhead at right), while a small fraction of episomal DNA in each lane migrated more slowly (Fig. 4D, lanes 6–8, 30–32, empty arrowhead at right). In contrast, most LANAΔ262-320 lanes (Fig. 4D, lanes 13–24, 33–38) had a substantially larger proportion of slower migrating episomes (Fig. 4D, empty arrowhead at right) when compared with faster migrating forms (Fig. 4D, solid arrowhead at right). This increased proportion of slower migrating DNA was most notable for LANAΔ262-320A (Fig. 4D, lanes 13–16) and LANAΔ262-320C (Fig. 4D, lanes 33–37), but also present for LANAΔ262-320B (Fig. 4D, lanes 17–20) and LANAΔ262-320Y (Fig. 4D, lanes 21–24). Therefore, consistent with earlier results (Fig. 3), LANAΔ262-320 again exhibited enhanced selection for larger, recombinant episomal forms.

To facilitate comparison of episome maintenance efficiencies for the different LANA mutants, we calculated the predicted number of cells necessary to seed per well to obtain 63.2% of wells with episome containing cells. Applying the Poisson distribution for the number of episome containing cells in a well, when the average number of cells per well in a microtiter plate is one, it is expected that 36.8% of wells will have no episome containing cells. The percent of G418 resistant cell lines that contain episomes (Fig. 1) was used to estimate the number of wells containing cells with episomes for LANA and each mutant in the limiting dilution outgrowth experiment in Fig. 4A. Nonlinear regression analysis was then used to determine the number seeded cells/well that are expected to yield 63.2% of wells positive for at least one episome containing cell. These values were 9, 8, 28, 37, 234, 209, and 43 for LANA, LANA306-320, LANAΔ288-320, LANAΔ262-320A, LANAΔ262-320B, LANAΔ262-320Y, and LANAΔ262-320C, respectively (Table 1). Comparison of the values of the mutants to that of LANA demonstrate wild-type function for LANAΔ306-320 and fold deficiencies of 3.0, 4.1, 25.8, 23.0, and 4.7 for LANAΔ288-320, LANAΔ262-320A, LANAΔ262-320B, LANAΔ262-320Y, and LANAΔ262-320C, respectively (Fig. 1). The average deficiency for the LANAΔ262-320 cell lines was 14.4. Therefore, LANAΔ288-320 and LANAΔ262-320 are both reduced for episome persistence, and LANAΔ262-320 is more severely deficient. Although reduced, the overall episome maintenance efficiency was still greater than the efficiency of integration, since BJAB cells had a fold deficiency of 83.3 compared to LANA.

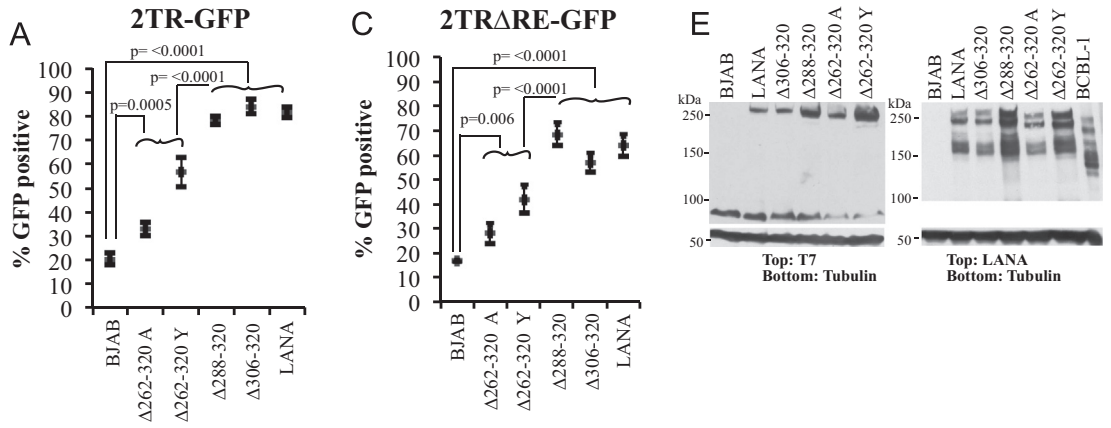
Table 1
Efficiency of episome maintenance.

Cell line	Predicted # cells/well necessary for 63.2% outgrowth of wells with episome containing cells
LANA	9
LANAΔ306-320	8
LANAΔ288-320	28
LANAΔ262-320 A	37
LANAΔ262-320B	234
LANAΔ262-320Y	209
LANAΔ262-320C	43

LANAΔ262-320 is modestly reduced for episome segregation

Since DNA segregation to daughter cell nuclei is an essential component of episome persistence, we assessed this function in the LANA mutants. N- and C-terminal LANA are essential for tethering KSHV DNA to mitotic chromosomes, and therefore are essential for segregation. We also recently showed that internal LANA residues 33-331 are critical for segregation (De Leon Vazquez et al., 2013). This work also showed that LANAΔ264-929 had an intermediate phenotype, consistent with amino acid 264 being near the border of sequence important for segregation. Since the mutants in this work all have deletions near the suspected border region important for episome segregation, we assessed DNA partitioning. BJAB or BJAB cells stably expressing LANA, LANAΔ262-320A, LANAΔ262-320Y, LANAΔ288-320, or LANAΔ306-320 were each transfected with equimolar amounts of p2TR-GFP or p2TR-ΔRE-GFP (kindly provided by Rolf Renne) (Skalsky et al., 2007). p2TR-GFP has two TR units and a GFP expression cassette. p2TR-ΔRE-GFP is identical to p2TR-GFP except it lacks the replication element (RE). Since p2TR-ΔRE-GFP contains the LANA binding sites, this plasmid can be used to determine the ability of LANA to segregate episomes in the absence of DNA replication. Eighteen to twenty hours after transfection with p2TR-GFP or p2TR-ΔRE-GFP, cells were sorted for GFP positive cells (since GFP expression indicates the presence of TR DNA), and seeded at 1.5×10^5 cells/ml. GFP expression and cell concentrations were then assessed daily by FACS.

Fig. 5A shows the percentage of GFP positive cells after transfection of p2TR-GFP, which is capable of both LANA DNA replication and segregation. In the absence of selection, TR DNA is expected to be lost from dividing cells, and LANA slows this loss (De Leon Vazquez and Kaye 2011b; Grundhoff and Ganem, 2004; Skalsky et al., 2007). Since the cells had slightly different rates of proliferation after GFP sorting, the GFP percentage was compared after a constant amount of cell proliferation at which point cells had amplified $e^{1.5}$ (4.48 fold) for each cell line, which generally occurred about 2–3 days after sorting, and was well within the approximate linear growth range for the cell lines. As expected, BJAB cells, which lack LANA, had a rapid rate of GFP loss, with a mean of only 21% of cells positive. In contrast, the GFP loss rate from LANA expressing cells was substantially slower, with a mean



B

p2TR-GFP Individual p-values

	BJAB	Δ262-320 A	Δ262-320 Y	Δ288-320	Δ306-320	LANA
BJAB	--	0.0168	<0.0001	<0.0001	<0.0001	<0.001
Δ262-320 A	0.0168	--	<0.0001	<0.0001	<0.0001	<0.001
Δ262-320 Y	<0.0001	<0.0001	--	0.0002	<0.0001	<0.001
Δ288-320	<0.0001	<0.0001	0.0002	--	0.2533	0.5196
Δ306-320	<0.0001	<0.0001	<0.0001	0.2533	--	0.6071
LANA	<0.0001	<0.0001	<0.0001	0.5196	0.6071	--

D

p2TRΔRE-GFP Individual p-values

	BJAB	Δ262-320 A	Δ262-320 Y	Δ288-320	Δ306-320	LANA
BJAB	--	0.0805	0.0006	<0.001	<0.0001	<0.0001
Δ262-320 A	0.0805	--	0.0317	<0.0001	0.0001	<0.0001
Δ262-320 Y	0.0006	0.0317	--	0.0003	0.0203	0.0016
Δ288-320	<0.0001	<0.0001	0.0003	--	0.0681	0.4537
Δ306-320	<0.0001	0.0001	0.0203	0.0681	--	0.2551
LANA	<0.0001	<0.0001	0.0016	0.4537	0.2551	--

Fig. 5. LANA amino acids 262–320 exert a modest effect on TR DNA segregation. (A) Ten million BJAB cells or BJAB cells stably expressing LANA or the different LANA deletion mutants were transfected with 5000 copies per cell (5×10^{10} total copies) of p2TR-GFP. Eighteen to twenty hours post-transfection, cells were sorted for GFP expression and seeded at similar cell concentration. GFP expression was monitored daily by flow cytometry for 14 days. Experiments are averages of four experiments. GFP expression was compared at the time point where cells had reached $e^{1.5}$ times the concentration from that of day 1 of cell seeding. Mean percent GFP positive is shown with bars indicating standard error. *p*-values for comparisons between indicated groups are shown. (B) Table shows *p* values for comparisons of individual cell lines in panel A. (C) Cell lines were assessed similar to in panel A but were transfected instead with p2TRΔRE-GFP, which lacks the RE elements in the TR sequence which are required for LANA mediated replication. GFP expression was assessed daily for 7 days. (D) Table shows *p* values for comparisons of individual cell lines in panel C. (E) Western blot of BJAB or BJAB cell lines expressing LANA or LANA mutants at the time of DNA segregation analysis. Left, top, anti-T7 epitope blot; left, bottom, anti-tubulin blot; right, anti-LANA blot. Brightness and contrast were uniformly adjusted within each panel with Adobe Photoshop. $\sim 3.5 \times 10^5$ cells were loaded per lane for the anti-T7 blot and tubulin blots, and $\sim 1.5 \times 10^5$ cells were loaded per lane for the anti-LANA blot.

of 82% cells GFP positive. LANAΔ288-320 and LANAΔ306-320 retained GFP similar to LANA and had means of 79% and 84% cells GFP positive, respectively. In contrast, LANAΔ262-320A and LANAΔ262-320Y were both reduced for the ability to retain GFP, with means of 33% and 57% GFP positive, respectively. The higher retention rate of LANAΔ262-320Y compared to LANAΔ262-320A is likely due to its higher protein expression level (Fig. 5E). Since 2TR-GFP can replicate and segregate, these results indicate that LANAΔ262-320 is deficient in DNA replication and/or segregation. Comparison of group A comprised of LANA, LANAΔ288-320, and LANAΔ306-320 with group B comprised of LANAΔ262-320A and LANAΔ262-320Y resulted in a *p* value of <0.0001. Individual *p* value comparisons for cell lines are shown in Fig. 5B.

In contrast to 2TR-GFP, transfection of 2TR-ΔRE-GFP assesses only DNA partitioning since the absence of the RE sequence abolishes LANA mediated DNA replication. As expected, 2TR-ΔRE-GFP was rapidly lost from BJAB cells and 17% had GFP expression (Fig. 5C). In contrast, LANA retained GFP with much higher efficiency with a mean of 64% of cells GFP positive. Similar to the results with p2TR-GFP, both LANAΔ288-320 and LANAΔ306-320 retained GFP similarly to LANA, with means of 69% and 57% GFP positive cells (Fig. 5C). Both LANAΔ262-320A and LANAΔ262-320Y were reduced for the ability to retain GFP, with means of 28% and 42% of cells GFP positive. The lower GFP retention rates of LANAΔ262-320Y and LANAΔ262-320A compared to LANA were not due to reduced protein expression as these mutants expressed at levels at least as high as LANA (Fig. 5E). These results indicate that the rate of 2TR-ΔRE-GFP loss was modestly reduced for LANAΔ262-320Y and moderately reduced for LANAΔ262-320A. The slightly higher rate of LANAΔ262-320Y GFP retention compared to LANAΔ262-320A may be due to LANAΔ262-320Y's higher level of protein expression. Comparison of group A (LANA, LANAΔ288-320, and LANAΔ306-320) with group B (LANAΔ262-320A and LANAΔ262-320Y) had a *p*-value <0.0001. Individual *p* value comparisons for cell lines are shown in Fig. 5D. Therefore, deletion of LANA residues 262-320 appears to modestly reduce episome segregation, while deletion of residues 288-320 or 306-320 does not.

LANAΔ262-320 is highly deficient for DNA replication

We assessed the LANA mutants for the ability to replicate TR DNA. BJAB cells or BJAB cells stably expressing LANA, LANAΔ306-320, LANAΔ288-320, or LANAΔ363-320 were transfected with p8TR-gB DNA, which contains eight copies of the KSHV TR element. p8TR-gB was isolated from Dam methylase bacteria and therefore is susceptible to DpnI digestion. However, after replication in mammalian cells, which lack Dam methylase, p8TR-gB is resistant to DpnI digestion. The amount of replicated, *Dpn*I resistant, p8TR-gB DNA was determined by real time PCR.

Fig. 6 shows the fold DNA replication for LANA and LANA mutants relative to BJAB control. LANA efficiently mediated DNA replication at 47.80 fold greater than the control BJAB cells. LANAΔ306-320 and LANA Δ288-320 also efficiently replicated DNA at 64.42 and 67.55 fold higher than control. The modestly higher levels of replication for LANAΔ306-320 and LANAΔ388-320 compared to that of LANA could be due to the higher levels of LANA expression for these mutants (Fig. 5B). In contrast, LANAΔ262-320A and LANAΔ262-320Y were dramatically reduced in their abilities to replicate p8TR-gB DNA and mediated replication at levels of only 4.93 and 5.05 fold over control, respectively; these levels were $\sim$10 fold less than that of LANA. These lower levels of replication were not due to reduced protein expression, since LANAΔ262-320A and LANAΔ262-320Y were each expressed

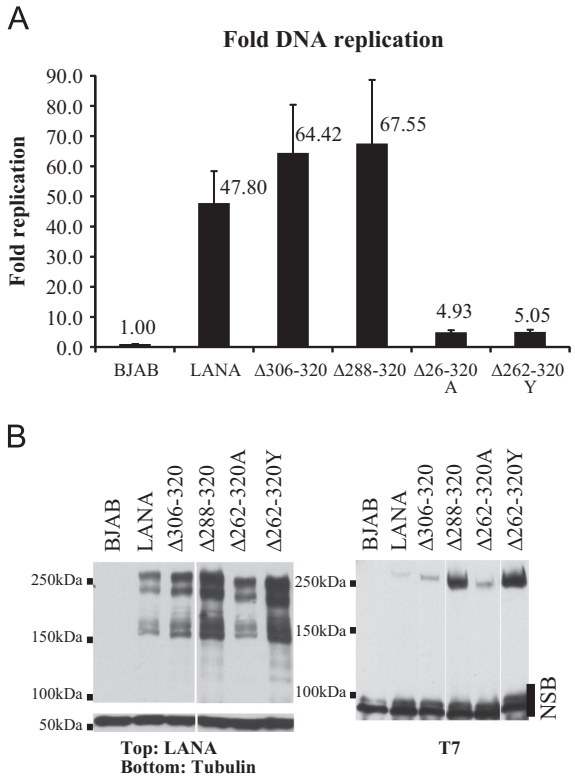


Fig. 6. (A) LANA residues 262–320 are critical for LANA mediated DNA replication. BJAB or BJAB cells stably expressing LANA, LANAΔ306-320, LANAΔ288-320, LANAΔ262-320A or LANAΔ262-320Y were transfected with p8TR-gB. Fold replication relative to the negative control BJAB is shown. Results are the average of four experiments and standard deviation is shown. (B) Western blot of LANA or LANA mutants from cell lines used for DNA replication assays. LANA was detected with anti-LANA or anti-T7 antibody. Bottom panel at left shows anti-tubulin blot. Brightness and contrast in individual panels were uniformly adjusted using Adobe Photoshop. Each lane contains $\sim 1.5 \times 10^5$ cells for the anti-LANA blot and $\sim 3.5 \times 10^5$ cells for the anti-T7 blot. NSB, non-specific band.

at higher levels than LANA (Fig. 5B). Therefore, LANAΔ262-320 is highly deficient for DNA replication.

Discussion

Previous work implicated the LANA region immediately upstream of the internal repeats as important for episome maintenance since LANAΔ264-929 was ~20 fold more deficient for episome persistence compared with LANAΔ332-929 (Fig. 1) (De Leon Vazquez et al., 2013). This work performed a targeted analysis of this region by investigating the function of LANAΔ306-320, LANAΔ288-320, and LANAΔ262-320. LANAΔ306-320 had wild-type episome maintenance efficiency, but LANAΔ288-320 and LANAΔ262-320 were reduced ~3.0 and ~14.4 fold, respectively. Although LANAΔ288-320 was not deficient in DNA replication or partitioning, it is possible that its moderately elevated protein expression level compared with LANA may have masked subtle defects in these assays that account for its ~3.0 fold episome maintenance deficiency. Importantly, LANAΔ262-320 was highly deficient for DNA replication but only modestly deficient for episome segregation, indicating that DNA replication deficit is the primary factor underlying its episome maintenance deficiency.

Previous work found that LANA residues 275-332, which overlap with the LANA sequence (residues 275-467) that interacts with the histone methyltransferase SUV39H1, were involved in releasing DNA from heterochromatic areas in both human and murine cells (Stuber et al., 2007). Since those residues overlap with the region identified as important for episome maintenance, we considered the possibility that the ability to alter chromatin might relate to LANA's episome persistence function. However, although we were able to replicate the finding of LANA's release of DNA from heterochromatic areas in murine L cells, our results suggest that this function localizes to three independent LANA regions located within LANA 1–32, LANA 33-331, and C-terminal LANA. Since the deletion in LANAΔ262-320 overlaps with LANA 33-331, it remains possible that a component of the episome maintenance deficiency might relate to a partial loss of ability to reorganize chromatin. The effect of sequence within LANA 1-32 on altering chromatin may be through its ability to bind the histone H2A/H2B acidic patch, which can induce nucleosome array compaction in vitro. In addition, a GFP fusion with LANA amino acids 1-32 induced alterations in chromatin as regions of Hoechst exclusion and focus formation in U2OS cells (Chodaparambil et al., 2007). It is possible that differences between the results here and those of Stuber (Stuber et al., 2007) may relate to differences in cell lines, transfection or staining conditions.

Notably, LANAΔ262-320 demonstrated enhanced selection for slower migrating episomal forms compared with LANA (Figs. 3 and 4). These forms migrate near genomic episomal BCBL1 DNA, which is ~200 kb, and is substantially larger than the input circular, covalently closed p8TR DNA. These slowly migrating forms are due to recombination events in p8TR DNA, including expansion of the TR elements and multimerization of the input p8TR plasmid, as discussed previously (Ballestas and Kaye, 2001; De Leon and Kaye, 2011a). It is likely that the increase in TR numbers, or amplification of key TR components compensates for LANAΔ262-320's deficiency for the ability to mediate episome persistence. Although such compensatory events due to recombination can readily occur in these episome maintenance assays, it is highly unlikely that KSHV can tolerate similar recombination events and remain viable. Therefore, the deficiency induced by deletion of LANA residues 262-320 may substantially impact the viability of KSHV. In fact, a recombinant KSHV containing a LANA mutant similar to LANAΔ332-929, which is 10.9 fold deficient for episome maintenance (Fig. 1) and therefore slightly less compromised than LANAΔ262-320, was incapable of establishing cell lines containing episomal virus (Alkharsah and Schulz, 2012).

Some differences in episome maintenance efficiencies for LANAΔ262-320 were evident when this mutant was expressed at different levels. LANAΔ262-320A and LANAΔ262-320C both expressed at levels similar to LANA and were reduced for episome maintenance 4.1 and 4.7 fold, respectively, while LANAΔ262-320B and LANAΔ262-320Y expressed at higher levels and were reduced 25.8 and 23.0 fold, respectively. Interestingly, LANAΔ262-320A and LANAΔ262-320C both appeared to allow more rapid selection of the slowly migrating, recombinant episomal forms compared with LANAΔ262-320B and LANAΔ262-320Y (Fig. 4D). This finding suggests that the more rapid evolution of larger episomes, which may contain recombination events that compensate for LANAΔ262-320 deficiencies, may be responsible for the increased episome maintenance efficiencies of LANAΔ262-320A and LANAΔ262-320C compared with LANAΔ262-320B and LANAΔ262-329Y. This finding also suggests that higher levels of LANAΔ262-320 slow selection of recombination events and indicates that LANA may directly interact with cell machinery involved in DNA recombination and higher LANA levels may interfere with recombination.

Results indicated that the deletion within LANAΔ262-320 has a small effect on segregation of episomal DNA. These findings are consistent with previous results (De Leon Vazquez et al., 2013) that showed that LANAΔ264-929 had a modest deficiency for segregation, whereas LANA deletions which extended further upstream induced severe segregation defects. Therefore, amino acid 262 is likely near the border of sequence critical for episome segregation.

This work demonstrates that LANA residues 262-287 are critical for DNA replication. LANAΔ306-320 and LANAΔ288-320 were wild-type for replication, while LANAΔ262-320 was reduced ~10 fold compared to LANA (Fig. 5). Since LANAΔ288-320 was expressed at a higher level than WT LANA, it remains possible that this mutant may be mildly deficient for replication but that its higher protein expression level compensated for the deficit. Both LANAΔ262-320A and LANAΔ262-320Y were similarly deficient for DNA replication, despite LANAΔ262-320Y protein expression at a substantially higher level, indicating that higher expression cannot compensate for the replication defect. The identification of this region as important for replication is consistent with previous results that showed a ~2 fold reduction in replication ability for LANAΔ264-929 compared with LANAΔ332-929 (De Leon Vazquez et al., 2013). Interestingly, LANAΔ262-320's replication of ~5 fold over control was similar to the ~4–5 fold over control level of replication when all internal LANA sequence was deleted and N- and C-terminal LANA were fused (De Leon Vazquez et al., 2013), suggesting that amino acids 262-320 exert a dominant role in LANA mediated DNA replication. However, other LANA regions also have roles in replication since internal deletions that did not involve residues 262-320 also reduce replication, although to lesser degrees (De Leon Vazquez et al., 2013).

It is likely that LANA residues 262-287 interact with a key cell factor critical for DNA replication. Since KSHV does not express DNA replication factors during latent infection, the virus is dependent on host cell machinery. LANA has been reported to interact with several proteins involved in replication. Origin recognition complex (ORC) proteins are essential for licensing DNA replication, and ORC 1-6 associate with LANA and are recruited to TR DNA in a LANA dependent fashion (Lim et al., 2002; Stedman et al., 2004; Verma et al., 2006). However, the ORCs bind C-terminal LANA, which is present in LANAΔ262-320, although conflicting results of weak (Verma et al., 2006) or no (Lim et al., 2002) binding of ORC1 with LANA 1-340 were reported. Topoisomerase IIbeta (TopoIIbeta) causes dsDNA breaks during DNA replication and a TopoIIbeta inhibitor, ellipticine, decreased LANA replication (Purushothaman et al., 2012). However, TopoIIbeta interacts with N-terminal LANA, which is present in LANAΔ262-320. LANA also interacts with structure-specific recognition protein 1 (SSRP1), which is a component of the chromatin modifying complex FACT (facilitating chromatin transcription) and knockdown of SSRP1 reduced LANA replication (Hu et al., 2009). The region of LANA interacting with SSRP1 has not yet been mapped. Ubiquitin specific protease 7 (USP7) has a repressive effect on LANA DNA replication, and USP7 interacts with C-terminal LANA (Jager et al., 2012). LANA also interacts with replication proteins A1 (RPA1) and A2 (RPA2), which bind ssDNA during DNA replication (Shamay et al., 2012). The LANA region with which RPA1 and RPA2 interact is not yet known. Therefore, it is possible that LANAΔ262-288 may recruit one of these or a different critical DNA replication factor to facilitate replication.

In summary, this work demonstrates that LANA residues 262-320 are critical for DNA replication and episome persistence. These residues are also likely near the border of sequence important for episome segregation. Further work is necessary to gain an understanding of the mechanisms mediating these functions.

Materials and methods

Cell lines

BJAB cells were maintained in RPMI medium containing 10% bovine growth serum (BGS) (Hyclone) or Fetalplex (Gemini) and 15 μg/ml gentamicin. KSHV-infected BCBL-1 cells were maintained in RPMI medium containing 20% BGS or Fetalplex and 15 μg/ml gentamicin. Cells stably expressing the different LANA mutants were grown in RPMI medium containing 10% bovine growth serum BGS (Hyclone) or Fetalplex (Gemini), 15 μg/ml gentamicin and 200 units/mL of Hygromycin B. Mouse L cells were grown in DMEM medium containing 10% BGS and 15 μg/ml gentamicin.

Plasmids

All T7 LANA constructs start at amino acid 4 and were cloned into pSG5 oligo (De Leon and Kaye, 2011a). We generated pSG5 oligo T7LANAΔ262-320, pSG5 oligo T7LANAΔ288-320 and pSG5 oligo T7LANAΔ306-320. Residues 321-331 were not deleted due to cloning difficulties engendered by the nearby repeat elements. pSG5 oligo-T7LANA (De Leon and E., Kaye, 2011a) was digested with *Eco*RV and *Xho*I and the LANA fragment was then ligated into pBluescript KS(−) and termed pBS LANA (*Eco*RV/*Xho*I). pSG5 oligoT7LANA contains the multicloning site from pEGFP-c1 that includes *XhoI* and *HindIII* sites. The 5′ portions of the LANA deletion mutants were amplified by PCR from pSG5 oligoT7LANA using the forward primer NotI T7 tag-F (ATAAGAATGCGGCCGCCCACCATGGCATCGATGACAGGTGGC) with the reverse primer Lana261AlaHinc-R (TCCCCCGTCAACTGCTG-CTGCTGCTGTAGGCGGTTGGCGTGGCGGAGTATCG), Lana287AlaHinc-R (TCCCCCGTCAACTGCTGCTGCTGCCGCAAGGAGCACCAACATGGC), or Lana305AlaHinc-R (TCCCCCGTCAACTGCTGCTGCTGCCTGATTATTTTC-GGAACATTCTTTTT). The reverse primers add four alanines followed by a *Hinc*II site. Due to a convenient unique *Hinc*II restriction site in LANA, deletions were made through residue 320. After amplification, the PCR fragment was digested with *Hinc*II, which digests near the 5′ and 3′ ends of the fragment, and cloned into the *Hinc*II-digested pBS LANA (*Eco*RV/*Xho*I) to generate pBS LANAΔ262-320, pBS LANAΔ288-320 and pBS LANAΔ306-320. The pBS LANA deletion constructs were each digested with *Hind*III, *Nru*I and *Bgl*I (*Bgl*I digests the pBS vector at multiple sites to simplify LANA purification). The LANA fragment was then inserted into the vector component of *Hind*III/*Nru*I digested pSG5 oligo T7LANAΔ332-929(De Leon Vazquez et al., 2013). pEGFP-C1-ENLS contains a nuclear localization signal cloned downstream of green fluorescent protein (GFP) in pEGFP-C1 (Clontech) (Hung et al., 2001). GFP LANAΔ33-888 contains LANA deleted for the indicated residues cloned downstream of GFP. To generate pEGFP-C1-ENLS-GFP-LANAΔ33-888, the XhoI/BamHI fragment (containing the LANA sequence) from pSG5oligo T7 LANAΔ33-888 (De Leon and Kaye, 2011a) was inserted into pEGFP-C1-ENLS after digestion with XhoI and BamHI. To generate pEGFP-C1-ENLS-LANA, pSG5oligo T7LANA was digested with AscI and NruI and the released LANA fragment was cloned into pEGFP-C1-ENLS-LANAΔ33-888 after digestion with AscI and NruI. pEGFP LANA1-331, pEGFP LANA 1-331 GMR, pEGFP-LANA 33-331 (De Leon Vazquez et al., 2013), GFP LANA1-32, GFP LANA GMR (Barbera et al., 2004), and GFP-LANA933-1162 (Kelley-Clarke et al., 2007a) were previously described. GFP-LANA779-1049 was generated by cloning the sequence from F-LANA1 779-1049 (Komatsu et al., 2004) downstream of GFP. All constructs were confirmed by sequencing. p8TR contains eight copies of the KSHV terminal repeat unit (TR) cloned into pRep9 (Invitrogen) which was modified by deleting the sequence between *Cla*I and *Kpn*I (Ballestas et al., 1999; Barbera et al., 2004). p8TR-gB is a modified version of p8TR which contains a small target sequence cloned into *Hind*III used to measure p8TR replication by real time PCR (De Leon Vazquez and Kaye 2011b). p2TR-GFP and p2TR-ΔRE-GFP were kindly provided by Rolf Renne(Skalsky et al., 2007). Each contains two copies of TR and also a GFP expression cassette. p2TR-ΔRE-GFP lacks the 32 bp replication element (RE) which is adjacent to the two LANA binding sites present in each TR; the RE is required for LANA mediated replication but not for binding of LANA to the TR. Therefore the plasmid can be used to investigate the ability of LANA to segregate TR-containing DNA in the absence of replication.

Generation of BJAB cells stably expressing LANA proteins

BJAB cells were transfected in 400 μl of RPMI medium at 200 V and 960 μF in a 0.4-cm-gap cuvette with a Bio-Rad electroporator (Ballestas et al., 1999). pSG5 oligo plasmids (70 μg) encoding T7LANA and the T7LANA deletion mutants were cotransfected with a plasmid carrying the hygromycin resistance gene downstream of a simian virus 40 promoter into BJAB cells (10 μg). After 48 h, cells were seeded into 96-well plates at 1000 cells/well and selected for hygromycin B resistance (200 units Hygromycin B/mL, Calbiochem). Clones resistant to hygromycin B were screened for LANA expression by Western blot and immunofluorescence.

Immunofluorescent microscopy

0.3×10^6 L cells were transfected with 1 or 2 μg of plasmid using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. After transfection, cells were grown on cover slips in 6-well plates in 1.5 mL DMEM containing serum. Fluorescence microscopy was performed 48 h after transfection. Transfected cells were washed with phosphate-buffered saline (PBS) and fixed for 10 min in 4% paraformaldehyde (Polysciences) in PBS. After fixing, cells were washed with PBS, treated with 0.5% Triton X-100 for 5 min and rinsed with PBS again. Cells were blocked for 1 h in 20% normal goat serum in PBS. To detect LANA or the LANA deletion mutants in the absence of GFP fusion proteins, cells were incubated in a humid chamber for 1 h with anti-LANA monoclonal antibody 1A2-12 (1:2500) (gift of Mary Ballestas) or anti-T7 epitope antibody (1:3500, Novagen). Cells were then incubated for 30 min with secondary anti-mouse Alexa 488 (1:2000, Molecular Probes) diluted in blocking buffer and washed three times with PBS. Cover slips were applied to slides after addition of Hoechst 33258 diluted at 1 μg/mL in Prolong Gold antifade solution (Molecular Probes) to detect DNA. Microscopy was performed using a Ziess AxioPlan 2 microscope.

DNA replication assay

To assess LANA mediated DNA replication, cells were transfected and DNA replication assessed by real time PCR quantification of DpnI resistant DNA 72 h after transfection as previously described (De Leon Vazquez and Kaye, 2011b) (De Leon Vazquez et al., 2013). To normalize for transfection efficiencies, the total amount of p8TR-gB present at 24 h post-transfection was divided by that in BJAB control, and termed the ratio for normalization. The replicated DNA at 3 days post-transfection was divided by the corresponding ratio for normalization to obtain the normalized replication of the particular test sample. For fold replication of each sample compared to BJAB control, the normalized replication of the each sample was divided by the normalized replication of BJAB control.

Selection of G418-resistant cells and Gardella gel analysis

BJAB cells and BJAB stably expressing T7LANA or T7LANA deletion mutants were transfected with 30 μg p8TR using a BioRad electroporator as above. After transfection, cells were seeded in 5 mL of medium without drug selection in a T25 flask (Fig. 3) or in one well of a 6-well plate (Fig. 4). We found that cells had better survival and recovery post-transfection when seeded into one well of a 6 well plate, so experiments in Fig. 4 were performed in this manner. Seventy-two hours post-transfection, LANA or LANA mutated protein expression was assayed by western blotting. Also after 72 h, transfected BJAB cells and BJAB cells stably expressing T7LANA or T7LANA deletion mutants were seeded in 96-well plates at 1000, 100, 10 or 1 cell/well in medium containing G418 (600 μg/ml) (Gemini) without hygromycin. GraphPad Prism was used to perform non-linear regression analyses with limiting dilution outgrowth values to compare episome persistence efficiencies. Gardella gel analysis was performed on G418 resistant clones by in situ lysis of cells in gel-loading wells with protease (Sigma) and sodium dodecyl sulfate (Gardella et al., 1984) followed by electrophoresis in Tris–borate–EDTA. DNA was transferred to a nylon membrane, and episomal DNA detected using a ^{32}P-labeled TR probe. Signal was detected by autoradiography.

Antibodies for Western Blots

LANA from whole cell lysates was detected by loading ~350,000 cells per lane in a Bio-Rad mini gel with a 15-well comb (7% SDS-PAGE). LANA proteins were detected with anti-T7 HRP conjugate antibody (Novagen 69048) at a dilution of 1:9000. Alternatively, LANA proteins are detected with anti-LANA human serum after loading ~100,000 cells for SDS-PAGE. Tubulin was detected with anti-alpha tubulin (Sigma T5168) at a dilution of 1:3000. Secondary HRP conjugated antibodies were diluted at 1:5000 (Santa Cruz).

TR DNA segregation assay

DNA segregation assays were performed as previously described (De Leon Vazquez et al., 2013). Briefly, ten million BJAB or LANA expressing BJAB cells in log-phase growth were transfected with 5×10^{10} copies (0.08 pmol) of plasmid p2TR-GFP (407 ng DNA), or p2TR-ΔRE-GFP (467 ng DNA) using Amaxa nucleofactor program O-17 in 150 μL of solution V. Eighteen to twenty hours post-transfection cells were sorted for GFP positive cells (BD FACS Aria sorter) and suspended in RPMI medium at a concentration of 150,000 cells/mL (day 0). Cell concentration was measured daily for the first five days by FACS using Count Bright Absolute Counting Beads (Invitrogen), and the percentage of GFP positive cells monitored daily and plotted. After reaching $\sim 1 \times 10^6$ mL^{-1}, cells were kept in growth phase by reducing concentration to $\sim 0.2 \times 10^5$ mL^{-1}.

Although the LANA and BJAB cell lines grew similarly, some minor differences in growth occurred after cell sorting. To compare the efficiency of episome segregation for the LANA mutants while also accounting for differences in cell growth rates, the percent of GFP positive cells was compared at a mutant-specific, plasmid-specific time point (denoted t^*_{sp}, s indexing LANA mutant strain, p indexing plasmid) at which the cell concentration reached $e^{1.5}$ (4.48) times the concentration measured at day 1 after cell seeding. A factor of $e^{1.5}$ was used since it was well within the approximately linear part of the growth pattern for all the transfected cells. In order to estimate the date after transfection at which cells reached $e^{1.5}$ times the concentration of day 1, growth curve models were fit to replicate data for each transfection. Repeated observations of GFP retention were made for replicates of each mutant, and we used quadratic logistic regression to model the relationship between the proportion of cells expressing GFP and the time elapsed from the initial transfection for each replicate transfection. For each combination of mutant–plasmid, the estimated proportion of cells that were expressing GFP at t^*_{sp} was obtained using replicate-specific logistic regression parameter estimates. Distributions of these growth corrected GFP expression rates are summarized as mean ±-standard error. To determine p values, for each plasmid and cell line, we computed the mean GFP percentage ± standard error at t^*_{sp} over the four replicates and compared the mean values among cell lines using the Fisher least significant difference method from a one-way ANOVA based on PROC GLM of the Statistical Analysis System (SAS) software version 9.3. In addition, p values were determined

for comparisons of group A composed of LANA, LANAΔ306-320, and LANAΔ288-320 with group B composed of LANAΔ262-320A and LANAΔ262-320C and for each group compared to BJAB using a similar one-way ANOVA based on three groups. All computations for the segregation analyses were performed using SAS version 9.3.

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